Comparison of the Effects of ORG 30029, Dobutamine and High Perfusate Calcium on Function and Metabolism in Rat Heart

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D. J. GRANDIS, G. A. MACGOWAN AND A. P. KORETSKY. Comparison of the Effects of ORG 30029, Dobutamine and High Perfusate Calcium on Function and Metabolism in Rat Heart. *Journal of Molecular and Cellular Cardiology* (1998) **30**, 2605–2612. Cardiac contractility may be enhanced via multiple cellular mechanisms resulting in varied effects on cardiac energetics. The mechanisms that account for the varied energetic responses are not well understood. The purpose of this investigation was to compare the effects of the calcium sensitizing agent ORG 30029 (*N*-hydroxy-5,6-dimethoxy-benzo[*b*]thiophene-2-carboximidamide hydrochloride, a calcium sensitizing agent which increases contractility without increasing calcium transients significantly), dobutamine and high perfusate calcium on contractility and energetics. Langendorff-perfused rat hearts were stimulated with ORG 30029, dobutamine and high perfusate calcium in graduated concentrations while myocardial oxygen consumption (MVO_2) and force-time integral were measured. ORG 30029, dobutamine and high perfusate calcium increased contractility in a dose-dependent manner. Despite an increase of 50% in systolic pressure and a 17% increase in force-time integral from control, ORG 30029 had no significant effect on MVO_2 at the lower concentrations ($n=6$). However, dobutamine ($n=4$) and high perfusate calcium ($n=4$) caused a 65% increase in systolic pressure and a 17% increase in force-time integral and a 50% and 41% increase in MVO_2 respectively ($P<0.05$). High energy phosphates (by ^{31}P NMR), and lactate production were unaltered by these agents, suggesting that metabolism was steady state. Basal metabolism tended to increase slightly with dobutamine but not with ORG 30029 or high perfusate calcium. ORG 30029, dobutamine, and high perfusate calcium increase contractility in perfused rat hearts with disparate effects on energetics. These differences may be accounted for, in part, by differences in energy expenditure for calcium handling. © 1998 Academic Press

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Introduction

Cardiac contractility may be enhanced via multiple cellular mechanisms with varied effects on cardiac energetics (Alpert *et al.*, 1992). When contractile force is increased by an elevation in the calcium transient (the rise in intracellular calcium concentration during depolarization) there is a proportionate rise in myocardial oxygen consumption (MVO_2) (Wu *et al.*, 1992). Contractility may also be increased by enhancing the "sensitivity" of the contractile proteins to calcium (Lee and Allen,

1997). Studies of the effects of calcium sensitizing agents on cardiac energetics have yielded various results. Some studies have shown that increasing calcium sensitivity increases contractility in a more energetically efficient manner than inotropic agents which increase the calcium transient amplitude (Aoyagi *et al.*, 1991, Groß *et al.*, 1993). However, other studies have not found any difference in efficiency (deTombe *et al.*, 1992 , Hata *et al.*, 1992).

We previously compared the effects of the unique calcium sensitizing agent EMD 57033, which does not affect calcium transients (Gambassi *et al.*, 1993,

Solaro *et al.*, 1993), to dobutamine in isolated rat hearts. In that study, despite a 40% increase in cardiac work, dobutamine increased MVO_2 47% while EMD 57033 did not cause any significant increase in MVO_2 (Grandis *et al.*, 1995). Dobutamine has β_1 agonist properties and may decrease calcium sensitivity (Ruffulo, 1987, Endoh and Blinks, 1989). Thus, the different effects of the two agents on calcium sensitivity could have caused the different energetic responses. Conversely, the energetic differences may be accounted for by differences in the energetic costs of calcium handling. Finally, because dobutamine shortened duration of contraction significantly and EMD 57033 did not, it was impossible to compare the effects on energetics of dobutamine and EMD 57033 at similar increases in force-time integral.

ORG 30029 (*N*-hydroxy-5,6-dimethoxy-benzo[*b*]-thiophene-2-carboximidamide hydrochloride) is a cardiotonic agent which increases contractility both by inhibition of phosphodiesterase activity as well as via calcium sensitizing (Cottney *et al.*, 1990). ORG 30029 has been demonstrated to increase contractility several-fold with minimal effects on calcium transients (Kawabata and Endoh, 1993). The purpose of this investigation was to compare the energetic effects of increasing contractility via graded concentrations of ORG 30029 to that of dobutamine and high perfusate calcium, both of which increase calcium transients significantly. High perfusate calcium concentration was employed because it is able to increase contractility without β_1 agonism or shortened duration of contraction, like dobutamine (deTombe *et al.*, 1992). At lower concentrations, ORG 30029 caused significantly less of an increase in MVO_2 than did dobutamine and high perfusate calcium. To probe possible mechanisms for this difference in MVO_2 at these concentrations, lactate production, phosphate metabolite concentrations (by ^{31}P NMR), and basal metabolism were determined with each agent.

Materials and Methods

Heart perfusion

Male Sprague–Dawley rats (Taconic Farms, Germantown, N.Y.) weighing 325–350 g were fasted overnight. Animals were anesthetized with 50 mg pentobarbital and anticoagulated with 1000 U heparin intraperitoneally. Hearts were excised and perfused within 15 s in the Langendorff manner (Neely *et al.*, 1967). Hearts were perfused at a constant perfusion pressure of 80 mmHg. Coronary perfusion pressure was measured via an in-line pressure transducer. Perfusate was not recirculated. To measure left ventricular pressure, a water-filled latex balloon attached to 3 cm non-compliant tubing was placed through the mitral valve into the left ventricle. Left ventricular pressure was determined using a pressure transucer (Gould, Cleveland, OH).

To assure a uniform heart rate in all studies, hearts were placed at 5 Hz using a stimulator and non-metallic pacing leads (KCl-agar) attached to the right ventricular apex. To eliminate autonomous beating, the atria was removed and the AV node was crushed. Left ventricular diastolic pressure was set at 8 mmHg pressure during the initial equilibration period by adjusting the intraventricular balloon volume. The mean volume of the balloon was 0.1 ± 0.04 ml. If the left ventricular systolic pressure did not reach 80 mmHg the heart was not used.

The perfusate was a modified Krebs–Henseleit buffer containing the following constituents: 115 mM NaCl, 4.5 mM KCl, 26 mM $NaHCO_3$, 1.8 mM $MgSO_4$, 2.0 mM $CaCl_2$, 0.1 mM NaEDTA, 10 mM pyruvate and 11 mM glucose (Neely *et al.*, 1967). KH_2PO_4 was excluded from the buffer to enable accurate measurements of intracellular inorganic phosphate (P_i) from ^{31}P NMR. Pyruvate was added to the buffer to maximize the redox state of the heart mitochondria and minimize the production of ATP by glycolysis (Kingsley-Hickman *et al.*, 1986). Perfusate osmolarity was determined (Wescor vapor pressure osmometer) to be between 285 and 295 mOsm. To eliminate the effects of catecholamine release by pacing, 0.1 μM esmolol (Dupont, Wilmington, DE) was added to the buffer. Perfusate temperature was maintained at 37°C via an in-line thermistor and heat exchanger. The perfusate was oxygenated by vigorous bubbling of the solution with a mixture of 95% O_2 and 5% CO_2. The pH of the perfusate was 7.40. At the end of each experiment, the hearts were removed from the cannula and weighed. Hearts were air dried for 2 days and weighed again for the dry weight.

Oxygen consumption

Myocardial oxygen consumption (MVO_2) was determined by the product of perfusate flow rate and the difference between afferent and efferent perfusate oxygen content. To avoid exchange between the effluent perfusate and ambient air, the heart was suspended in a small, closed glass chamber. Effluent dripped off the heart but hearts were

suspended in effluent. Samples were obtained at the site of exit of the effluent from the glass chamber. Oxygen content was measured with either a blood gas analyzer (ABL-30, Copenhagen, Denmark) or oxygen electrode (YSl, Yellow Springs, OH). Oxygen consumption was calculated as μmol/min/g dry weight of heart.

^{31}P NMR spectroscopy

^{31}P NMR measurements were made using a 4.7 Tesla, 40 cm horizontal bore Bruker Biospec NMR spectrometer (Bruker Inst., Billerica, MA) which operates at a phosphorus frequency of 81 MHz. ^{31}P NMR spectra were obtained from the isolated heart in a closed chamber which was suspended and centered in a 2 cm diameter solenoid radio frequency coil tuned to the ^{31}P frequency. Spectra were acquired using a 90° pulse and an 8 s repetition time. Eighty acquisitions were averaged; requiring 10.7 min of acquisition time. The areas of the peaks were determined using the Bruker integration method.

The following procedure was used to determine the relation between peak areas and the concentration of metabolites. In a separate set of experiments, the degree of saturation with the protocol above was determined by obtaining spectra both in the fully relaxed state (15 s) and with an 8 s repetition time. Based on HPLC analysis, the concentration of ATP in hearts was determined to be 24.7 μmol/g dry weight (Grandis *et al.*, 1995). The peak areas of phosphocreatine (PCr) and P_i were compared to the peak area of the β ATP peak. Using the known [ATP] from high performance liquid chromatography (HPLC) as the value of the β ATP peak and the respective saturation factors for ATP, PCr and P_i, the concentrations of [PCr] and [P_i] were determined.

All chemical shifts were determined relative to PCr which was assigned a shift of 0 ppm. Intracellular pH was determined as previously described from the chemical shift of the P_i peak and [Mg^{2+}] was determined from the chemical shift difference between the α and β ATP peaks (Koretsky *et al.*, 1989). Free [ADP] was determined assuming the creatine kinase catalyzed reaction was in equilibrium (Veech *et al.*, 1979): [PCr], [ATP], Mg^{2+}, and pH were determined by ^{31}P NMR and HPLC (From *et al.*, 1986). An average total creatine ([PCr] and [Cr]) was determined to be 62.1 μmol/g dry weight by HPLC. The equilibrium constant for the creatine kinase reaction, K_{app}, was calculated for each heart, accounting for pH and [Mg^{2+}] (Lawson

and Veech, 1979). Knowing these parameters, [ADP] was determined using the formula: [ADP] = [ATP]·[total creatine-PCr]/[PCr]·K_{app}; where [ATP] and [ADP] represent the sum of all possible ionic species.

Two to three ^{31}P NMR spectra were obtained for each heart during the control steady-state period and inotropic state. The values for [PCr], [ADP], [P_i], pH and [Mg^{2+}] from spectra during each period were averaged for the control period and each inotropic agent. [ATP] and [TOTAL CREATINE] were determined by HPLC as previously described.

Lactate production

Lactate production was determined by measuring the difference in lactate concentrations between the perfusate entering and exiting the heart. Lactate concentration was determined using a standard commercial assay (Sigma Chemical Co., Kit #735, St Louis, MO). Three measurements were made during control period and during the infusion of the agents.

Experimental protocol

Throughout the entire experiment, left ventricular pressure was continuously measured while MVO_2 lactate production, and ^{31}P NMR spectra were obtained every 8–10 min. A control steady-state was established when three consecutive measurements of the ^{31}P NMR spectra, MVO_2, and pressure did not vary by more than 10%. This period was approximately 35 min in duration. In three separate experiments, no inotropic agents were infused and demonstrated that cardiac function, MVO_2 and ^{31}P NMR spectra were stable in this model for over 120 min (data not shown).

After a control steady-state was established, the perfusate was changed to the same perfusate with an inotropic agent added at a particular concentration. Each experiment used only one agent at one concentration per study. ORG 30029 was kindly donated by Organon Pharmaceuticals. It is relatively insoluble in water and thus was disolved in DMSO (Fisher, Pittsburgh, PA). The DMSO solution was added to the perfusion buffer to yield a final DMSO to water concentration of 1:1800. In a separate set of experiments, DMSO alone was added to buffer at a concentration of up to 1:1000 and had no effect on cardiac metabolism or function (data not shown). Dobutamine (Eli Lilly, Indianapolis, IN) and calcium were added directly to

Table 1 Functional and Energetic Effects of ORG 30029, dobutamine and perfusate calcium

	Concentration	Number	Systolic pressure (mmHg)	Diastolic pressure (mmHg)	Force–Time Integral (kdyne·sec)	Coronary Flow (ml/min)	MVO$_2$ (μmol/min/g dry wt)
Control		26	90 ± 8	6 ± 3	17 ± 3	22 ± 6	33 ± 7
ORG 30029							
	0.25 mM	6	136 ± 13*	4 ± 4	24 ± 3*	20 ± 3	34 ± 8
	0.375 mM	6	152 ± 12*	3 ± 4	29 ± 4*	23 ± 8	45 ± 4*
Dobutamine							
	60 μg	4	157 ± 18*†	3 ± 3	21 ± 5§	27 ± 7	52 ± 12*†
	75 μg	3	183 ± 8*†§	0 ± 0	24 ± 7	26 ± 5	57 ± 4*†
[Ca^{2+}]							
	4 mM	3	151 ± 20*¶	1 ± 2	26 ± 1*	27 ± 1	48 ± 4*
	6 mM	4	167 ± 8*†#§	1 ± 2	33 ± 3*†#	35 ± 5*†	64 ± 9*†§

Data presented as means $\pm$ standard deviation. $P<0.05$; * vs control, † vs 0.25 mM ORG 30029, § vs 0.375 mM ORG 30029, # vs 60 μg dobutamine, ¶ vs 90 μg dobutamine.

the buffer. Another steady-state was achieved with each agent and data was acquired for approximately 35 min.

Basal metabolism

To determine the effects of these inotropic agents on basal metabolism, rat hearts were perfused at constant pressure with the perfusate and conditions above while left ventricular pressure and MVO$_2$ were monitored. After a steady-state was achieved, hearts were arrested by changing the perfusate to one that was the same as control perfusate but contained 20 mM KCl. This concentration was chosen because it is the concentration which was found to suppress all autonomous beating of the heart in the presence of dobutamine. After an arrested steady-state was achieved, the inotropic agents were added to the perfusate as described above.

Data analysis

The force–time integral was derived as an estimation of cardiac mechanical work. It was determined from the pressure tracings above by digitally scanning the cardiac cycle from onset of contraction to the next onset of contraction (AdobePhotoshop, Mountain View, CA) and integrating the total left ventricular pressure over time. The diastolic portion of the cardiac cycle was included in the determination of the force–time integral because myocardial energy consumption has been demonstrated to be determined by ventricular pressures during both systole and diastole

(Suga, 1990). The force–time integral was derived by the formula: mmHg/seconds/surface area of the balloon·1329 dyne/cm^2. To calculate balloon surface area, it was assumed that the balloon was spherical. The force–time integral was expressed in kilodynes·seconds (kdyne·secs).

The functional, metabolic, and ^{31}P NMR data obtained in the control steady-state period from all experiments were pooled. Analysis of variance for repeated measures were used to determine differences within study populations and a *post hoc* Bonferroni/Dunn "t" test was used to test for significant differences between groups. Data are expressed as means $\pm$ standard deviation. The number of experiments are shown in the Tables.

Results

ORG 30029, dobutamine, and high perfusate calcium each increased systolic pressure and force–time integral in a concentration-dependent manner (Table 1). Each caused a downward trend in the diastolic pressure. However, despite a 50% increase in systolic pressure and a 17% increase in force-time integral compared to control, ORG 30029 had no significant effect on MVO$_2$ at the lower concentrations. This effect on MVO$_2$ differed from that of dobutamine and high perfusate calcium where a 65% increase in systolic pressure and a 17% increase in force–time integral compared to control resulted in a 50% and 41% increase in MVO$_2$, respectively. Developed pressure was effected in a similar manner to force-time integral by the three agents (Fig. 1). The different effects on MVO$_2$ between the agents was less significant at higher concentrations. Coronary flow tended to increase
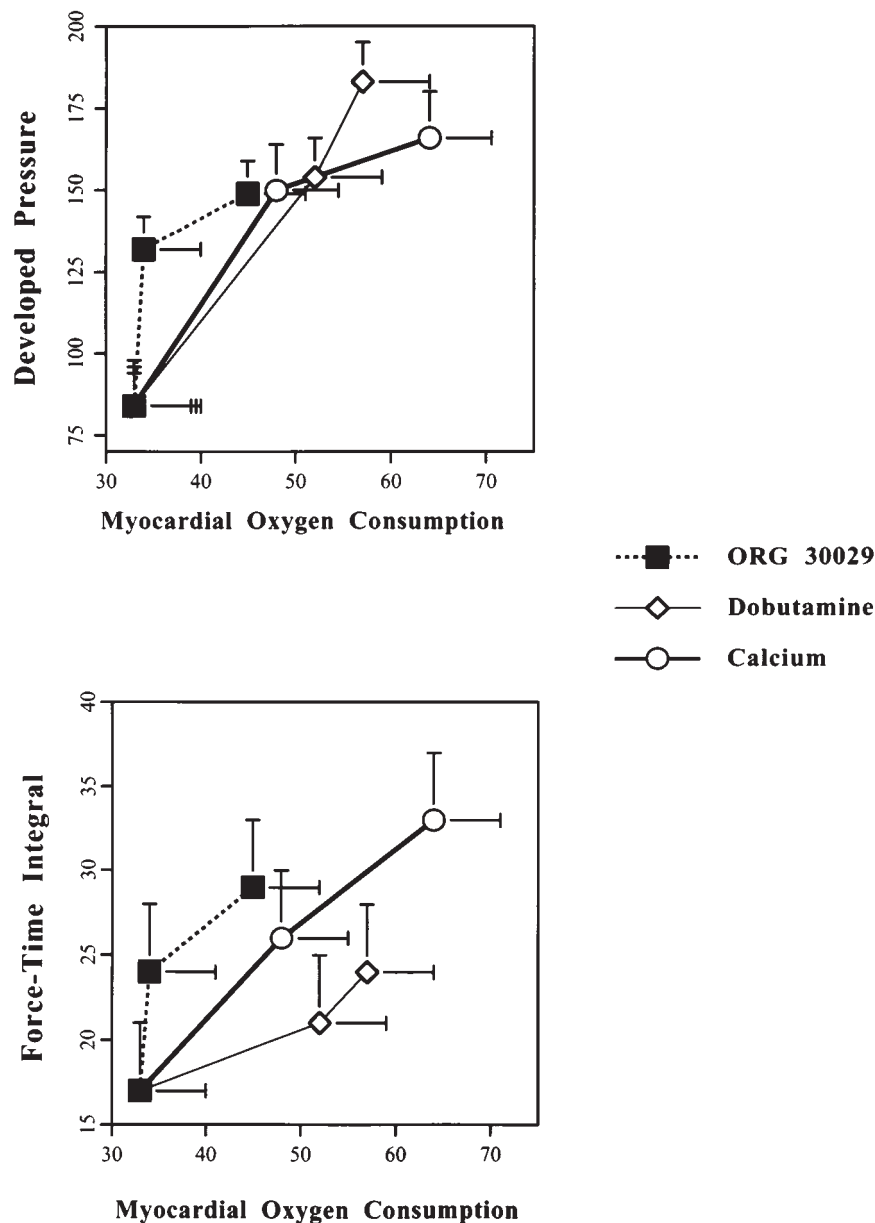
Figure 1 Relationship between changes in developed pressure and myocardial oxygen consumption (top), and force–time integral and myocardial oxygen consumption (bottom) for dobutamine, high perfusate calcium, and ORG 30029 in rat hearts. Dobutamine caused a greater increase in myocardial oxygen consumption for a similar change in force-time integral than did calcium. Both agents caused a greater rise in myocardial oxygen consumption than did ORG 30029.

with 6 mM perfusate calcium but not 4 mM perfusate calcium, dobutamine, or ORG 30029.

To elucidate possible mechanisms for the differences in contractile economy between the inotropic agents, high energy phosphate concentrations, lactate production, and basal metabolism were determined in hearts perfused at the concentrations that caused the largest differences in contractile economy. High energy phosphate concentrations in hearts were determined in a subset

of the experiments presented in Table 1 with ^{31}P NMR. As shown in Table 2, there were no significant differences in the phosphate metabolite concentrations; indicating that the production and utilization of high energy phosphates were in a steady-state. Likewise, lactate production was unaffected by perfusion by each agent. Basal metabolism, as measured in the KCl arrested heart, was not significantly altered by each inotropic agent (Table 3) though there was a trend towards an

Table 2 Effects of ORG 30029, dobutamine and calcium on high energy phosphate concentrations, pH_i, $[Mg^{2+}]$, and lactate production

	Control	ORG 30029	Dobutamine	Calcium
Concentration		0.375 mM	60 μM	6 mM
Number	9	3	4	2
[PCr] (μmol/g DW)	54 ± 7	51 ± 8	51 ± 7	46
[ADP] (μmol/g DW)	0.02 ± 0.02	0.03 ± 0.03	0.03 ± 0.02	0.05
[P_i] (μmol/g DW)	15 ± 13	13 ± 11	22 ± 15	17
pH_i	7.02 ± 0.10	6.94 ± 0.15	6.92 ± 0.15	7.00
$[Mg^{2+}]_i$ (mmol/l)	0.8 ± 0.3	0.7 ± 0.4	0.6 ± 0.3	0.6
lactate (mmol/l)	0.08 ± 0.05	0.05 ± 0.05	0.8 ± 0.04	0.12

Data presented as means $\pm$ standard deviation. there were no statistically significant differences between the agents with respect to the parameters measured. DW, dry weight.

Table 3 Effects of ORG 30029, dobutamine and calcium on basal metabolism in the KCl-arrested rat heart (mean values)

	n	Baseline developed pressure	Baseline MVO_2	KCl arrest pressure	KCl arrest MVO_2	Agent+KCl pressure	Agent+KCl MVO_2
ORG 30029	2	82	36	14	17	11	17
Dobutamine	2	87	40	19	19	26	24
Calcium	2	80	36	17	17	16	14

increase in both MVO_2 and pressure with dobutamine.

Discussion

The present investigation demonstrated significant differences in energetic responses to increased contractility in perfused rat hearts between the calcium sensitizing agent ORG 30029, the β_1 agonist dobutamine, and high perfusate calcium. These differences resulted in divergent effects on cardiac energetics. The effect of dobutamine and high perfusate calcium on cardiac function, metabolism, and phosphate metabolites by ^{31}P NMR have been well described previously (Kingsley-Hickman *et al.*, 1992). The responses seen in this study are very similar to previous reports. To our knowledge, the simultaneous effects of ORG 30029 on MVO_2, phosphate metabolites, and function have not been investigated.

The energetic effects of ORG 30029 seen in the current investigation are consistent with those seen in other trials with other calcium sensitizing agents. Gross *et al.*, demonstrated a similar difference between the calcium sensitizing agent EMD 57033 and dihydroouabain in guinea pig hearts (Gross *et al.*, 1993). This result was consistent with our comparison of EMD 57033 and dobutamine in rat hearts (Grandis *et al.*, 1995). However, other studies

have not found a difference in efficiency between calcium sensitizing agents and conventional inotropic agents. A study comparing the effects of pimobendan and dobutamine on MVO_2 in dog hearts found no significant difference in the efficiency of contraction between these two agents, though economy increased 14% with pimobendan (Hata *et al.*, 1992). Another study in dog hearts demonstrated no energetic differences between EMD 53998 (another calcium sensitizing agent) and calcium infusion (de Tombe *et al.*, 1992). The conflicting results between these studies may be due to differences in species used, pharmacological actions of the agents, or experimental methods employed. The energetics of the isovolumic heart differ from that of the ejecting heart (Alpert *et al.*, 1992., Suga, 1990). Thus, it is uncertain whether the marked differences in cardiac energetics observed between agents in this investigation would be found in the ejecting heart as well.

What are possible mechanisms for the disparate responses in MVO_2 between the different inotropic agents employed in the current investigation? Neither lactate production or high energy phosphate concentrations were altered in hearts stimulated with these agents. Thus, alterations in these parameters cannot account for the different effects on MVO_2 by these inotropic agents. Though basal metabolism tended to increase slightly with dobutamine, the 25% increase in MVO_2 with

dobutamine in the KCl-arrested heart cannot fully account for the 50% increase in MVO_2 in the beating heart. Furthermore, high perfusate calcium did not affect MVO_2 in the KCl-arrested heart but did increase MVO_2 in the beating heart by 41%. It is possible that these agents altered the P:O ratio in mitochondria. However, this ratio has been demonstrated to be unaffected in rat hearts stimulated with dobutamine (Kingsley-Hickman et al., 1992).

Some investigators have proposed that calcium sensitizing agents like ORG 30029 enhance the myosin–actin interaction during contraction by increasing the rate of cross-bridge cycling (Lee and Allen, 1997). It is conceivable that this enhancement may create an improvement in the force generated per mole ATP hydrolyzed. β_1-Adrenorecepter agonists such as dobutamine have been demonstrated to decrease calcium sensitivity in mammalian hearts (Endoh and Blinks, 1989). High perfusate calcium may also diminish calcium sensitivity via calmodulin-mediated inosotol 1,4, 5-triphosphate suppression of calcium sensitivity (Demaille and Pechère, 1983). Thus, ORG 30029 may have increased contractile economy as compared to control, dobutamine, or high calcium via energetic enhancement at the myosin–actin level. Similarly, dobutamine may have decreased contractile economy as compared to control via β_1 adrenorecepter agonism.

Alternatively, different effects on calcium transients by these agents may also account for the differences seen in energetics. At the lower concentrations (like those employed in the current investigation) ORG 30029 has been demonstrated to increase cardiac contractility with minimal effects on calcium transients (Kawabata and Endoh, 1993). At higher concentrations, however, ORG 30029 increases phosphodiesterase activity and increases calcium transient amplitude (Cottney et al., 1990, Kawabata and Endoh, 1993). β_1-Adrenorecepter agonists such as dobutamine and high perfusate calcium increase calcium transients several-fold (Endoh and Blinks, 1989, Kawabata and Endoh, 1993). Because an increase in calcium transient amplitude would increase energy expenditure for calcium handling, it is also tenable that ORG 30029 would cause less of an increase in MVO_2 than dobutamine and high perfusate calcium for a similar increase in contractility. It has been estimated that calcium handling utilizes 25–45% of the energy consumed by the beating mammalian heart (Suga, 1990). The data in the current study are consistent with this estimation.

In summary, ORG 30029 increased MVO_2 less than dobutamine and high perfusate calcium for a similar increase in force–time integral. These changes occurred without affecting high energy phosphate concentrations, lactate production, or basal metabolism. The energetic differences may be accounted for by differences in calcium handling or effects on the myosin–actin interaction.

Acknowledgments

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